FINAL REPORT

TO THE

OFFICE OF NAVAL RESEARCH

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Title of Project: Biochemical and Genetical Research on Certain Mutants of Neurospora

Summary of Work Accomplished During Course of Contract

Objective and General Approach: The central objective of the work was to investigate closely a number of biochemical mutants of Neurospora with the aim of discovering the nature of their genetic blocks. A number of different approaches were used – all of which have the same final objective. (1). Based on the premise that internal inhibitions caused by an over-supply of certain metabolic constituents are possible or even probable, two mutants which grow on minimal medium, but are inhibited by specific amino acids, were investigated with the aim of establishing the nature of the inhibition. It is believed that such an approach will lead to a better understanding of the interactions in metabolism and hence provide the means for further work on mutants which demonstrate definite requirements for supplements to minimal medium. (2). The investigation of mutants which show definite requirements for specific compounds, but are inhibited when other compounds are added along with the required supplements. (3). Since all biochemical mutants show differences from wild type besides their requirement for specific compounds, attempts were made with considerable success to put some of these differences on a more chemical basis than had heretofore been the case. Specifically the approach was to determine the intracellular free amino acid content of the mycelium of a number of different strains in order to determine whether their amino acid patterns were different from one another and wild type. (4). Crosses were made between certain types of biochemical mutants to determine whether metabolic inbalances in the single mutants would not prove to be complementary in the double mutant which would therefore be a balanced individual. These crosses were made primarily to test hypotheses concerning the nature of the metabolic disfunctions in the parent strains, and were predicated on the assumption that if the double mutants grew better than the parent strain the metabolic blocks of the parent strains had been partially or completely relieved in the combinations.

Summary of Accomplishments: The work accomplished will be described here in four parts corresponding to the four general approaches discussed above.

1. Mutants inhibited by amino acids, but which otherwise grow well on minimal medium: Two mutants were studied, T77 which is inhibited by threonine, and T66, inhibited by histidine.

T77 is a temperature mutant which requires thiazol for optimal growth at temperatures below 25°C. Actually at 18° little or no growth is attained unless thiazol is added. At these temperatures thiazol may be replaced by a combination of methionine or homocysteine and threonine. When grown at 30°, the mutant has a wild type phenotype. It has no requirements on minimal, and it is not inhibited.

The threonine inhibition is exhibited at 35°C at pH 5.0. In addition to L-threonine, other compounds also cause inhibition. These are listed in Table 1.

Various compounds relieve these inhibitions of growth completely or partially. Table 2. presents a summary of these compounds and their effects in the presence of the inhibitors, L-threonine and α-~~amino~~ KETO butyric acid.

A thorough study of the metabolism of the T77 mutant is being made to determine in what way it is different from the wild type. It was first attempted to examine the threonine dehydrase activity of the mutant to determine whether this activity was higher than in the wild type. Despite initial results reported in the affirmative in previous reports, it is now established that this activity in the mutant appears to be identical to wild type.

An examination of the culture filtrates of the mutant when growing in the presence of L-threonine showed that the keto acids, pyruvic acid, α-keto-β-methylvaleric acid and α-keto isovaleric acid accumulated in large amounts in the medium. These compounds do not

Table 1.

Compounds which Inhibit T77

L-threonine	
α-ketobutyric acid	
choline	partially
glycine	partially

Table 2.

Summary of Inhibition Relief at 35° C

Compound Tested	Compounds Relieved	
	L-threonine	-ketobutyric acid
L-methionine	++	0
DL-methionine	++	0
DL-homocysteine	++	0
choline	+	++
choline + thizaol	++	±
DL-homoserine	++	0
acetaldehyde	0	+
glycine	0	+
glycine + thiazol	++	0
acetaldehyde + thiazol	0	0
L-valine	+++	+
D-valine	0	0
α -keto-β-ethylbutyric	0	0
α -keto-β-methylbutyric	0	0
acetic acid	0	0
pyruvic acid	0	0
DL-valine	0	0
DL-isoleucine	0	0
L-isoleucine	0	0
D-isoleucine	0	0

accumulate when L-threonine is absent, nor do they do so in the medium of wild type growing in the presence of threonine, except in trace amounts during the early phases of growth.

It was also found that Neurospora including both the mutant and wild type convert threonine to acetaldehyde and glycine.

Combining these data with what is known concerning the metabolism of threonine from other sources, the scheme shown in Figure 1. may be derived with a considerable degree of certainty that it is accurate in most respects.

It will be noted from Figure 1. that in the presence of threonine, a precursor for isoleucine (as proven recently by other workers with C^{14}) and a possible precursor for valine (as indicated by data from others and our laboratory), there is a block formed between these amino acids and their keto analogues which results in the accumulation of these precursors as well as the possible additional precursor of valine, pyruvic acid.

It was found that the inhibition of threonine could be completely relieved by L-valine, but not DL-valine or D-valine. This relief of inhibition is competitive (see Table 3.) and is much more effective than any hitherto reported relief found with other compounds such as methionine etc. It is also of interest to note that methionine, homocysteine, and homoserine relief is obtained with the DL forms, but valine is effective only as the L-form. DL-methionine is as effective as L-methionine. Surprisingly, no form of isoleucine relieves the inhibition to any consistent measurable degree.

A method was developed for the separation and quantitative determination of the three keto acids accumulated by T77 in the presence of threonine. By means of it, it was found that they do not accumulate to as high a degree when the mutant is grown in the presence of both threonine and the compounds which relieve threonine inhibition. This is particularly true of the keto analogue of valine, α-keto isolvaleric acid which is present

Figure 1.

Scheme for Threonine Metabolism in Neurospora

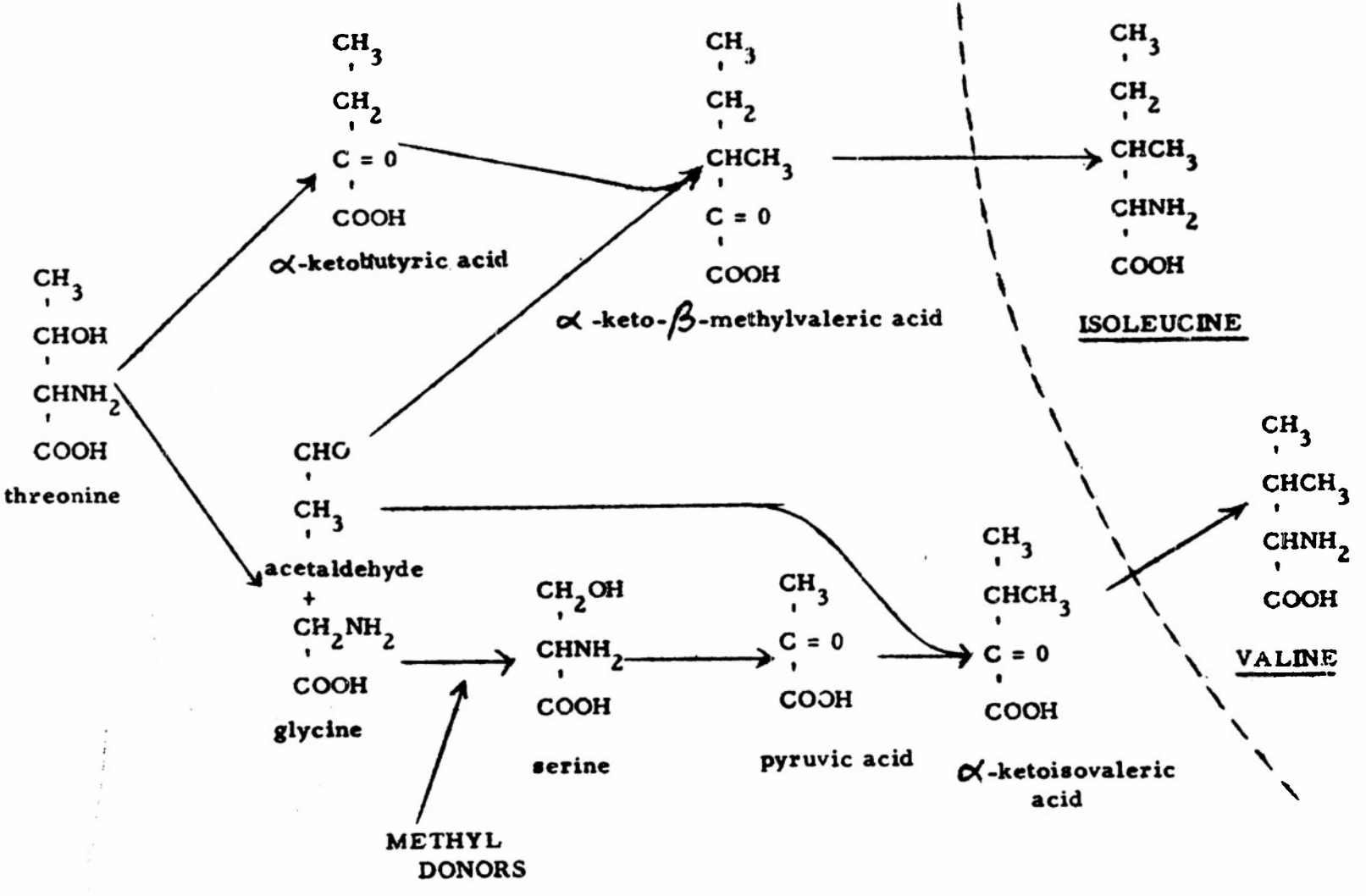

Table 3.

Competitive Effect of L-valine on L-threonine Inhibition

Concentrations of L-valine
those which give 1/2 maximal relief of threonine inhibition

L-threonine $M \times 10^{-3}$	L-valine $M \times 10^{-3}$	Inhibition Index
0.4	0.22	1.82
2.0	1.2	1.67
4.0	2.4	1.67

in extremely low concentration in the medium even in the presence of valine.

The present data point to the following metabolic interpretation of this mutant. L-threonine by some mechanism at present unknown causes an inhibition of the conversion of the α-keto analogues of isoleucine and valine to their corresponding amino acids. Actually this inhibition-block may be more apparent than real in the case of α-keto-β-methyvalerate, since isoleucine seems to be formed in the presence of threonine. Otherwise both isoleucine and valine would be required to relieve the threonine inhibition. The block between α-keto isovalerate and valine, at any rate, appears to be effective enough to cause a requirement for valine. The effect of methionine, choline, etc. as relievers of inhibition is tentatively suggested now to result from the donation of methyl groups by these compounds which would then bring about the formation of enough α-keto isovaleric acid to force the production of valine. This hypothesis would be particularly applicable if it could be shown that competition for a common transaminase between α-keto-β-methylvalerate and α-keto isovalerate is true in Neurospora as has been shown for E. coli.

The mutant inhibited by L-histidine, T66 has not been studied to as great an extent as in the example given above. Histidine inhibition is relieved by all amino acids tested with the exception of aspartic acid, lysine, proline, and hydroxyproline. Histidine in the mutant appears to upset nitrogen metabolism in a way which does not occur in wild type.

2. Inhibition in mutants with blocks in aromatic amino acid synthesis: Preliminary work with several mutants of Neurospora requiring aromatic compounds for growth involved an extension of the conventional screening technique used for identification of new mutants. It consisted of supplying a low concentration of the previously identified requirement for a strain and superimposing a high concentration of several complex mixtures on this supplement. By this method, mutants with a nutritional requirement

plus a sensitivity to any one of the components of the mixture can be detected.

Several mutants of such a class were investigated. The tyrosine requiring strain, T-145, studied most extensively, was shown to be inhibited by 20 amino acids normally considered to be universally present in all cells. The inhibition was competitive with tyrosine and the inhibition indices (Table 4.) ranged from 5-250. Five amino acids (Table 4.) showed no inhibition even when tested at a very high concentration. These compounds have a single characteristic in common. They are each regarded as "poor transaminators" or their transamination is somewhat unusual. It has only been recently that transamination was demonstrated with three (arginine, ornithine, and lysine) of these amino acids and in this case only under special experimental conditions. The remaining two (proline and hydroxy-proline) being imino acids would not be expected to utilize the usual transamination enzyme systems.

It is interesting also that two closely related compounds to an inhibitory amino acid do not act as inhibitors. Both phenylpyruvic and phenyllactic acids show no inhibition even at very high concentrations whereas their α-amino analog phenylalanine is a very potent inhibitor having an inhibition index of eight. Thus, it would appear that the essential functional group in causing inhibition is the α-amino group.

In light of the characteristics of the two groups of amino acids the keto analog of tyrosine was synthesized and tested. The synthetic compound served not only to replace the tyrosine requirement of the mutant but reversed the inhibition in a non-competitive manner. Thus, the keto analog of tyrosine was able to bypass inhibition by amino acids in a tyrosine requiring strain of Neurospora. This has been taken to indicate that some new and as yet unexplained essential function exists for the keto analog of tyrosine (p-hydroxyphenylpyruvic acid).

Several of the known oxidation products of p-hydroxyphenylpyruvic acid (homogentisic acid and 2,5 -dihydroxyphenylpyruvic acid) proved to be

Table 4.

Summary of the Inhibition Indices of the Amino Acids with T-145

0-10	10-20	20-40	60-80	150-250
leucine	alanine	glycine	citrulline	aspartic acid
methionine	serine (DL)	histidine	kynurenine	cysteine
phenylalanine	norleucine (DL)	isoleucine		glutamic acid
tryptophan	∝-phenylglycine (DL)	threonine		
norvaline (DL)		valine		
∝-aminobutyric acid (DL)				

Non Inhibitory

arginine
lysine
ornithine
proline
hydroxyproline

Note: All L-isomers except where indicated.

inactive in the system discussed above.

The essential nature of the keto analogs of aromatic amino acids is further supported by work with other mutants. Strains requiring phenylalanine for growth have been inhibited in a similar manner to T-145 by amino acids and non-competitive reversal is accomplished by the respective keto analog, phenylpyruvic acid.

This proposed essential function of keto analogs could not be only that of precursor of the amino acids, which has heretofore been contended, because interconversion of these two compounds appears to be blocked. Much data supports the major role of the amino compound rather than the keto in biosynthesis however, function of a keto moiety on a specific site in a protein is not inconceivable.

The factual accounts discussed above serve well to illustrate that the nutritional requirements of a mutant are only a gross indication of the multiple effects radiating out from a seemingly simple modification in metabolism.

3. Analysis of free intracellular amino acids by Neurospora: The free intracellular amino acid pool in Neurospora represents a metabolic phase of development in the organisms nitrogen metabolism. Ammonia nitrogen from the medium is taken up by the cells and converted to amino acids, which are subsequently built up into cell proteins. To study these metabolic processes, we used the Koch McMeekin modification of the Micro Kjeldahl method for ammonia nitrogen, and the alpha-amino nitrogen method of Moore and Stein (J.B.C. 176: 373, 1948). Protein nitrogen was determined after acid hydrolysis by the same methods. Color produced in these reactions was measured quantitatively with the Klett spectrophotometer.

Neurospora EM 5256A and EM 5297a were grown on a chemically defined medium containing 6.5 mg of ammonia nitrogen. Daily analysis of nitrogen indicated the rapid disappearance of ammonia nitrogen from the medium, its take-up and conversion to amino-substances inside the cell with an equilibrium resulting at least in quantity between the free

intracellular and the medium amino-nitrogen. At five days of growth both external and internal free amino nitrogen substances had reached their peak of about 3.25 mg and 4.25 mg of amino acids respectively. Close at about the same time the ammonia nitrogen in the medium had all been used up; and as the proten nitrogen still increased slowly up to between 9 to 15 days; free amino acids in and outside the cells decreased to a low at 9 days. About 40 mg of protein amino acids per flask constituted maximum growth. Dry mycelial weight of EM 5256A began to decrease after 6 days, and of EM 5297a to stay at a peak from the 9th to the 13th day. These results indicated the importance of the free intracellular amino acid pool in any study of development and genetics of Neurospora. In previous studies of three days and nine days old cultures, analysis of free intracellular amino acid extracts showed extensive differences between different strains of mutants and wild types. By the use of chromatographic methods some 32 ninhydrin-sensitive substances were encountered in these free intracellular cell extracts.

Analysis of 5 day old cultures was carried out with several mutants and especially with 36104 a methionine-less and 34A adenine-less. The later strains were back-crossed to wi'd type for five generations, and then again analyzed. All methionine-less mutants contained a larger amount of threonine and glutamine than either wild types or other mutants. As a matter of fact, threonine was even higher in concentration than alanine which normally represents the largest quantity of amino acid produced. Serine was present in large quantities in all these methionine-less mutants except the F_5 of opposite mating type to the parent strain. This represents an amino acid not linked to the mutant character as the other previously mentioned substances. Two other as yet unknown amino substances were also found in the mutants but not in wild types. A double mutant 239A adenine-methionine-less had all the characteristics of both the methionine and adenine types of mutants. In adenine-less mutants one of the same

unknown substance as in methionine-less mutants was present. They also lacked glutathione which is present in both wild types. A mutant T 145 A Tyrosine-p-hydroxyphenyl-pyruvic-less, produced a tremendous large quantity of alanine, almost twice as much as wild type, and the same is true for some of its other amino acids, although most had the usual quantity. A vitamin-less mutant T8 is peculiar in its production of what seems to be taurine, and a large amount of methionine. These are only a few examples of qualitative and quantitative differences encountered in the free intracellular amino acid fraction of Neurospora. Further work will certainly result in establishing metabolic pathways for many of these mutants since differences of the type encountered are due to basic genetic differences, and are closely linked to enzymatic and genetic control of growth and metabolism.

4. Crosses between biochemical mutants and results: Despite a considerable number of crosses made between different mutants very little positive information has been obtained from them.

Some interesting results were obtained from crosses between T77, the threonine inhibited mutant and certain threonineless mutants. One such double mutant (T77, 44104) gave growth responses to threonine equivalent to the parent threonine requiring strain at concentrations of threonine which completely inhibit the growth of T77 alone. In this case the 44104 gene mutation suppresses the T77 phenotype. However, another double mutant (T77, 35423) was even more markedly inhibited by threonine than T77.

PERSONNEL

The support provided by this contract has been of immense aid in making it possible to train scientists in biochemical genetics at the University of Texas. All four of the assistants to the principal investigator have or will receive their Ph. D. degrees on work accomplished under terms of this contract.

Dr. Charles O. Doudney received his Ph. D. in Genetics and Biochemistry in June, 1954. The title of his thesis is "Metabolic Interactions and Antagonisms of the Threonine Inhibited Strain of Neurospora crassa". He is now employed in the Division of Biology of the Oak Ridge National Laboratory in a research position.

Dr. A. Gib DeBusk received his Ph. D. degree in Genetics and Biochemistry in August, 1954. The title of his thesis was "Genetic and Biochemical Analysis of Aromatic Amino Acid Metabolism in Neurospora". He is currently employed by the Genetics Foundation of the University of Texas in a research capacity.

Mr. James B. Ragland and Mr. Robert Fuerst will both receive their Ph. D. degrees in January of 1955.

PUBLICATIONS AND REPORTS

1. Semi Annual Progress Report, June 13, 1953

2. Annual Progress Report, January 25, 1954

3. Doudney, C. O. and R. P. Wagner. A relationship of homocysteine metabolism to thiamin, serine and adenine biosynthesis in a mutant strain of Neurospora. Proc. Nat. Acad. Sci. 39: 1043-1052 (1953).

4. DeBusk, A. G. and R. P. Wagner. p-hydroxyphenylpyruvic acid function in Neurospora crassa. J. Am. Chem. Soc. 75: 5131 (1953).

5. Wagner, R. P. An apparent metabolic block induced in a Neurospora mutant by threonine. Records Gen. Soc. Amer. 23: 73 (Abstr.)

6. Ragland, J. B. A strain of Neurospora inhibited by histidine. Records Gen. Soc. Amer. 23: 61 (Abstr.)

7. Fuerst, Robert. Differences in free intracellular amino acids in Neurospora. Records Gen. Soc. Amer. 23: 41 (Abstr.)

3. Doudney, C. O. Gene interaction and temperature response of the threonine inhibited strain of Neurospora. Records Gen. Soc. Amer. 23: 38 (Abstr.)

Five manuscripts are now in the course of preparation. Copies of these will be provided as soon as they are completed and ready to be sent to journals for publication. Copies of the above abstracts are provided with this report.